IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: EL-HAJ & DOBSON
SERIAL NO.: 10/518,956
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TITLE: METHOD OF MAGNETICALLY MANIPULATING A CELL WITH MAGNETISABLE PARTICLES

EXAMINER: Ian D. Dang
ART UNIT: 1647

Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 CFR 1.132

1. I, Dr. Alicia Jennifer Hafeeza EL HAJ, am Professor of Cell Engineering and Director of the Institute for Science and Technology in Medicine at Keele University, United Kingdom (the assignee of the subject patent application), and an inventor of the subject-matter disclosed and claimed in the present application. I have a Ph.D. in the field of bioengineering and am the author of over 100 articles in peer-reviewed journals.

2. I understand the Examiner has acknowledged that the specification of the present application is enabling for (1) up-regulation of osteopontin in response to a magnetic field in the presence of anti-TREK antibody bound to magnetic nanoparticles binding to TREK channel in mesenchymal cells, and (2) the production of cartilage matrix proteins in mice by implanted human mesenchymal stem cells in the presence of magnetic nanoparticles bound to cells via an anti-TREK antibody in response to time varying magnetic fields.

3. I understand that Examiner has indicated the specification not to reasonably provide enablement for:

(A) an *in vitro* method for the generation of cartilage tissue from mammalian cartilage cells expressing mechanosensitive TREK potassium ion channels, and

(B) a method of the generation of new cartilage tissue in a patient, wherein the new cartilage tissue is generated from cartilage cells expressing the mechanosensitive TREK potassium ion channels.

4. I understand that Examiner determined Exhibit B from my earlier declaration (filed 08 January 2008, herein Declaration No.1) to evidence production of cartilage matrix proteins in mice by implanted human mesenchymal cells in the presence of magnetic nanoparticles bound to cells via an anti-TREK antibody.

5. I also understand that Examiner is of the view that insufficient evidence has been provided for a method of generation of cartilage tissue using magnetisable particles bound to an anti-TREK antibody, because the synthesis of cartilage matrix proteins is distinct from the production of cartilage tissue, and that Examiner indicated that Exhibit B does not provide any evidence for the presence of a ground substance rich in proteoglycan and elastin fibers.

6. I am aware that in reply to the Office Action of 25 November 2008 claims 175 and 186 are amended to refer to generation of cartilage tissue from chondrocyte progenitor cells.

7. I can now report the results of further studies carried out under my supervision, which address the lack of evidence the Examiner has indicated. These studies are described in Exhibit 1 and Exhibit 2, which are appended to this declaration.

8. Exhibit 1 describes *in vitro* studies using human bone marrow stromal cells (HBMSC) or human mesenchymal stem cells (hMSCs) labeled with magnetisable particles tagged with antibodies specific for the TREK-1 potassium ion channel. As described, following incubation of the cells and magnetisable particles in the magnetic force bioreactor there was a significant increase in expression of Collagen I, Sox 9, Cbfa 1 and osteopontin (Opn). This is consistent with the results shown in Exhibits A and B of my earlier declaration (Declaration No.1), but extends the finding of upregulation of these matrix proteins to HBMSCs, which are another type of chondrocyte progenitor cell.

9. Exhibit 1 continues to describe "3D studies". In these studies HBMSCs labeled with magnetisable particles tagged with antibodies specific for the TREK-1 potassium ion channel were subjected to magnetic conditioning as described. HBMSC labeled with TREK-1 antibody tagged magnetic particles showed elevated expression of **proteoglycan** synthesis around the cells accompanied by increased expression of Type-1 Collagen.

10. Exhibit 2 describes new tissue formation *in vivo* from HBMSCs labeled with TREK-1 antibody tagged magnetisable particles. New chondrogenic tissue formation was demonstrated that is superior to HBMSC alone and which approaches that observed when HBMSC are treated with TGF-β3.

11. Whilst unlabelled HBMSC showed negligible expression of proteoglycan or collagen, HBMSCs labeled with TREK-1 antibody tagged magnetisable particles showed elevated expression of proteoglycan and collagen matrix synthesis, including high levels of Type-1 and Type-2 collagen.

12. Examiner has said that cartilage tissue requires the presence of chondrocytes, a ground rich substance rich in proteoglycan and elastin fibers. No source of, or evidence for, this particular definition of cartilage tissue is provided in the Office Action of 25 November 2008. We have shown in Exhibits 1 and 2 that chondrocyte progenitor cells (HBMSCs and hMSCs) can be used *in vitro* (Exhibit 1) and *in vivo* (Exhibit 2) to cause elevation of cartilage matrix protein expression and elevation of proteoglycan synthesis around the cells by following the methods of claims 175 and 186 respectively. We did not test for the presence/absence of elastin but, in my opinion, this is not determinative of the formation of cartilage and does not detract from the clear indicatlon that new chondrogenic tissue formation is occurring both *in vitro* and *in vivo*.

13. In summary, here and in my earlier declaration (Declaration No.1), using the methods claimed we have shown:

(a) up-regulation of osteopontin in mesenchymal cells (Exhibit A, Declaration No.1),
(b) significant increase in expression of cartilage matrix proteins Collagen I, Sox 9, Cbfa 1 and osteopontin (Opn) *in vitro* from chondrocyte progenitor cells (Exhibit 1 and paragraph 8 herein),
(c) production of cartilage matrix proteins *in vivo* in mice from implanted human mesenchymal stem cells (Exhibit B, Declaration No.1),
(d) elevated expression of proteoglycan synthesis around chondrocyte progenitor cells *in vitro* (Exhibit 1 and paragraph 9 herein),
(e) elevated expression of proteoglycan and collagen matrix synthesis around chondrocyte progenitor cells *in vivo* (Exhibit 2 and paragraph 11 herein),
(f) New chondrogenic tissue formation from chondrocyte progenitor cells *in vivo* (Exhibit 2 and paragraph 10 herein).

14. As such, the evidence provided indicates that the generation of cartilage tissue from chondrocyte progenitor cells *in vitro* and *in vivo* according to the methods of claims 175 and 186 (as amended) can be performed without placing the skilled person under an undue burden of experimentation.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date 15 April '09

By: .....
Alicia El-Haj, Ph.D.

Additional Experimental Data – Exhibits 1 and 2

Exhibit 1. In vitro Modelling and Characterization of expression of genes involved in differentiation of human bone marrow and human mesenchymal cells using a magnetic conditioning bioreactor system.

Monolayer studies:

Human bone marrow stromal cells (HBMSC) were isolated from bone marrow aspirate (Lonza, UK) by adherent culture on tissue culture plastic. HBMSCs and hMSCs were incubated with antibody-coated nanoparticles at a concentration of 25µg of particles per $2x10^5$ cells overnight in serum free media. After acclimatisation, the magnetically labelled cells with between 5 & 15 internalised particles per cell were ready for use in the experimental setting at passage five. After washing, the cells labelled with magnetic nanoparticles and the control non particle-labelled hMSCs and HBMSCs, were seeded at a density of $1x10^4$ cells per cm^2 in 6-well plates in 2 ml of complete DMEM per well for activation in monolayer. The experimental test groups were exposed to a 1Hz cyclical loading using a magnetic force bioreactor (Figure 1) for one hour every other day for one week in monolayer. The controls included cells labelled with magnetic nanoparticles with/and without magnetic stimulation. The results demonstrated that when comparing for mRNA expression levels in HBMSC of the particle control with the tagged particles exposed to a magnetic field group, there was a significant increase (Student's t-test, $p \leq 0.05$) in Coll I, Sox 9, Cbfa 1 & Opn expression. However, when comparing all three groups (one way ANOVA, $p \leq 0.05$) the observed differences in mRNA expression were significant only in Coll I, Sox 9 & Opn. An increasing trend but varying in magnitude of mRNA expression was also observed in Coll I, Sox 9, Cbfa 1 & Opn in cells from a further 4 patients samples.

3D studies:

In addition, 3D encapsulated cell studies were completed. Adult human marrow was obtained from haematological normal patients undergoing routine elective hip replacement surgery (under approval of the Southampton & South West Hampshire LREC). HBMSC cultures were maintained in basal medium at 37°C in 5% CO_2. Prior to labelling the cells with the magnetic particles the HBMSC were labelled

with Cell Tracker Green/Ethidium Homodimer-1 (CTG/EH-1) to determine cell viability/cell necrosis respectively over the period of the experiment. 250nm silica magnetic particles (Micromod) were covalently bound to the anti-TREK antibody. These particles (Figure 1A) were bound to the HBMSC in culture for 24 hrs (Figure 1B) washed and encapsulated in 2% alginate-chitosan capsule and either placed in basal culture media in a six well plate (n=6 capsules) or subcutaneously implanted into male MF-1nu/nu severely compromised immunodeficient mice. We demonstrated after 24 hrs that magnetically labelled HBMSC were viable with little evidence of cell death.

Confident in the ability to label and modulate skeletal populations with magnetic nanoparticles, HBMSC were encapsulated into the alginate-chitosan capsules. Of the encapsulated cell capsule constructs, 4 capsules were used for subcutaneous implantation *in vivo* (cells from 4 patients) and 3 capsules per group were used for the *in vitro* experiments (cells from 2 patients). We initially looked at HBMSC cells; HBMSC cells labelled TREK-K^+ particles; TREK particles only; HBMSC + TGF-β3 (10ng/ml) (non exposure to the magnetic fields). The encapsulated HBMSC were placed in a six well plate and exposed to the magnetic conditioning bioreactor (Figure 1) at intermittent pulsing for 1hr daily intervals, at 1Hz frequency and 1-100pN/particle for 28 days 3 times a week for 21 days (see Figure 1D). For all in vivo studies, mice were placed in magnetic box and exposed to a magnetic field for 1hr 3 times a week for 21 days (Figure 1E). As in Figure 2, cells remained viable over the 21 days (Figure 2B) and extensive vascularisation was observed in the in vivo sub-cutaneous samples (Figure 2C and D) using cells labelled with TREK- K^+.

The unlabelled HBMSC encapsulated 3D *in vitro* group showed negligible expression of proteoglycans, collagen or Type-2 collagen (Figures 3A, E, M) but demonstrated weak expression of Type-1 collagen (Figure 3I). TGF-β3 stimulation of HBMSC resulted in the increased expression of proteoglycans (Figure 3B) with weak expression of Type-1 collagen (Figure 3J) and very strong expression of Type-2 collagen (Figure 3N). HBMSC labelled with TREK-K^+ magnetic particles encapsulated group showed some elevated expression of proteoglycan synthesis around the cells (Figure 3C) with increased expression of Type-1 collagen (Figure 3K) and negligible expression of Type-2 collagen (Figure 3O).

Exhibit 2. Demonstration of new tissue formation In vivo

In vivo Activation experiments:

In vivo studies were carried out over 3 weeks as detailed in Exhibit 1 for the capsule experiments.

Human bone marrow stromal cells (HBMSCs) containing magnetic nanoparticles (TREK-K+ and RGD) demonstrated new chondrogenic tissue formation *in vivo* superior to cells alone and approaching tissue formation observed with cells treated with TGF-β3.

In addition, we observed good vascularisation of implanted capsules in the in vivo studies.

Thus on analysis, unlabelled HBMSC showed negligible expression of proteoglycans or collagen (Figures 4A & E). HBMSC stimulated with TGF-β3 showed an increased expression of proteoglycans and collagen (Figures 4B & F) where the cells showed a ribbon-like migratory pattern. HBMSC/TREK-K^+ magnetic particles encapsulated group showed elevated expression of proteoglycan and collagen matrix synthesis (Figures 4C & G) and HBMSC labelled with RGD magnetic particles encapsulated group showed low level expression of proteoglycans and elevated matrix production of collagen (Figures 4 D & H) as well as the ribbon like migratory pattern. (Figures 4 D & H) similar to that of the MSC stimulated with TGF-β3.

As well as analysis for proteoglycans and collagenous proteins, we also examined the *in vivo* samples for specific expression of type I and type II collagen. The unlabelled HBMSC encapsulated group showed negligible expression of Type-1 collagen (Figure 5A) but displayed expression Type-2 collagen. HBMSC stimulated with TGF-β3 showed increased levels of expression of Type-1 collagen (Figure 5B) and Type-2 collagen (Figure 5F). HBMSC+TREK-K^+ magnetic particles encapsulated group showed high levels of Type-1 and Type-2 collagen expression (Figures 5C & G).

After 21 days blood vessels were found to surround the capsules (Figure 6A) that were subcutaneously implanted into MF-1nu/nu mice and exposed to the magnetic

fields. The HBMSC magnetic labelled particles showed some tubule vessel formation around the implanted capsules as well as penetration of cells into the capsules.

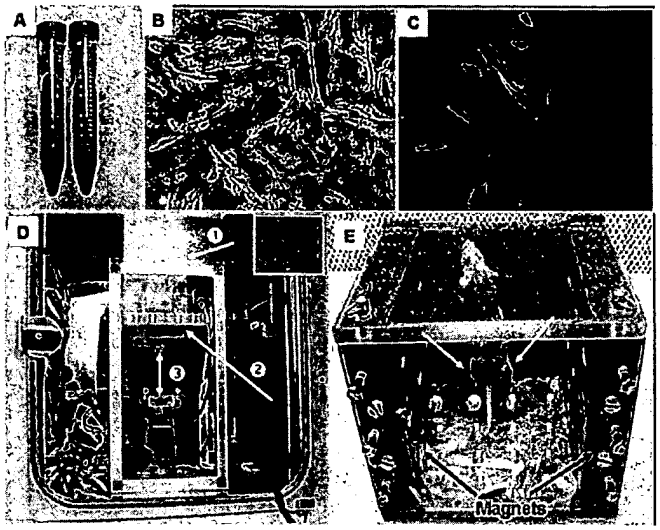

Figure 1. TREK- K^+ and RGD magnetic particles in solution (A), HBMSC labelled with magnetic particles (5-15 particles/cell) (B), Labelled HBMSC viability after 24hrs (cell tracker green/ Ethidium homodimer-1) (C). A pulse magnetic conditioning bioreactor (D) showing a six well plate containing the alginate/chitosan-magnetically labelled HBMSC (arrow ①) exposed to a pulsing magnetic field (arrow ②) which moved up and down (arrow ③) for 1 hour daily (Monday, Wednesday and Friday) intervals, at 1Hz frequency and 1-100pN/particle for 21 days. A magnetic box (E) where mice that had the encapsulated capsules subcutaneously implanted (white arrows) were exposed 1 hour daily (Monday, Wednesday and Friday) for 21 days.

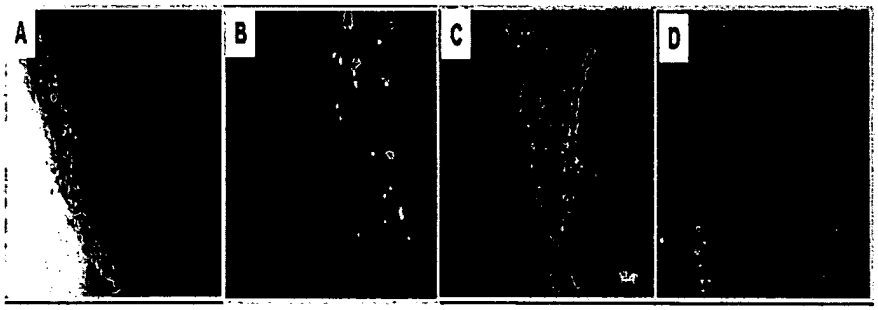

Figure 2. HBMSC encapsulated in capsules (A) Cell viability of HBMSC encapsulated in capsules (CTG/EH-1) (B) 21 days post subcutaneous implantation. Vessels surrounding the capsules with HBMSC +TREK- K^+ particles (C) and HBMSC + RGD particles (D) 21 days subcutaneous implantation.

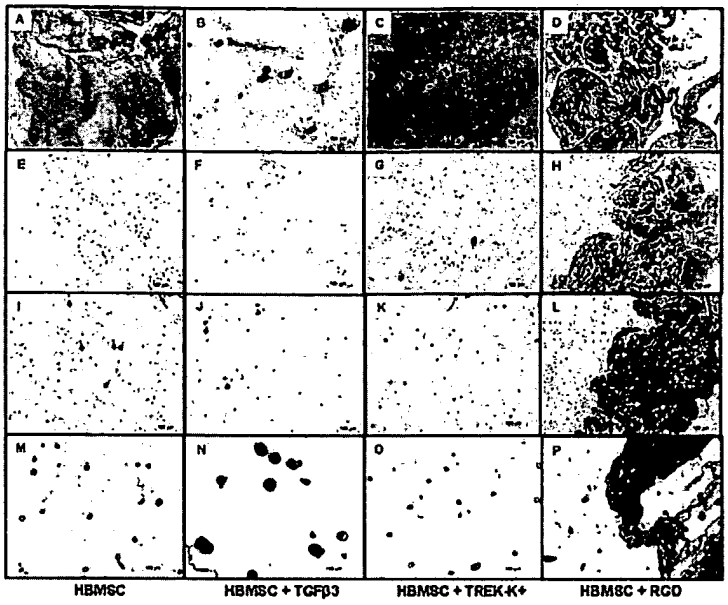

Figure 3. Histology and immunohistochemistry of HBMSC with and without labelled TREK-K$^+$ or RGD particles encapsulated in alginate/chitosan capsules and exposed to a pulsed magnetic field for 21 days. 6μm tissue sections stained for alcian blue/Sirius red (A-D); Golders' trichrome (E-H); Type-1 collagen (I-L) and Type-2 collagen (M-P).

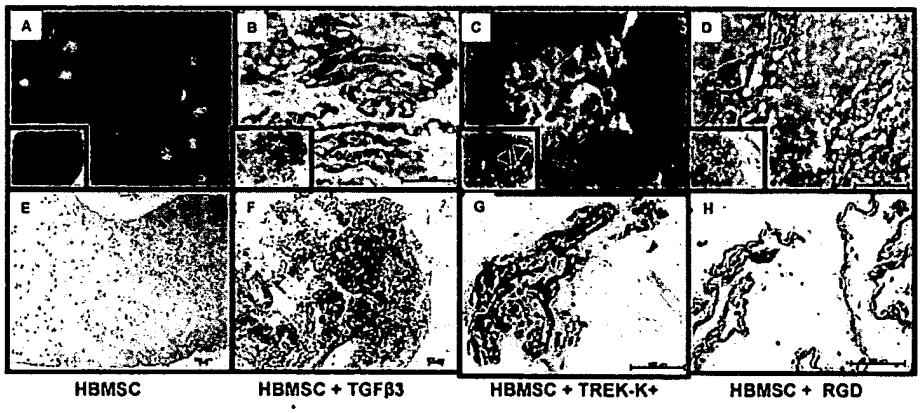

Figure 4. Histology of HBMSC with and without particles encapsulated in alginate/chitosan capsules subcutaneously in MF-1 nu/nu mice and exposed to a magnetic field for 21 days. 6μm sections stained for alcian blue/Sirius red (A-D) and Golders'trichrome(E-H) Representative lower magnification images of the capsules after 21 days.

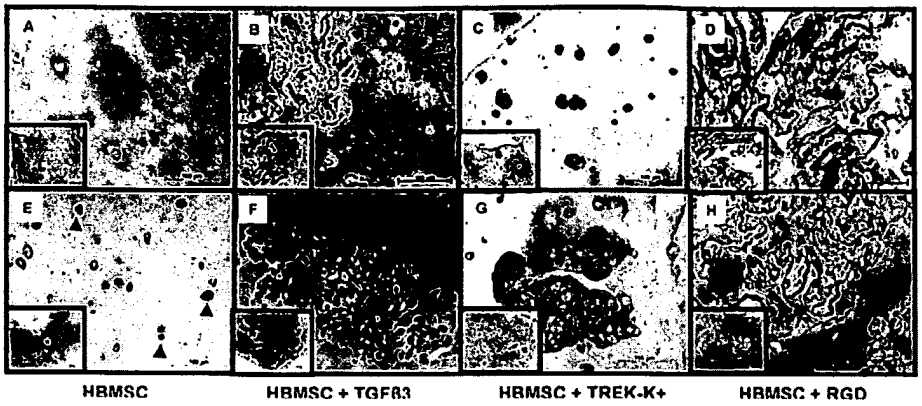

Figure 5. Type-1 and Type-2 collagen immunohistochemistry of HBMSC with and without labelled particles encapsulated in alginate/chitosan capsules implanted subcutaneously in MF-1 nu/nu mice and exposed to a magnetic field 1hr a day (M, W, F) for 21 days. 6μm tissue sections stained for Type-1 collagen (A-D) and Type 2 collagen (E-H) Representative lower magnification images of the capsules after 21 days (inset).

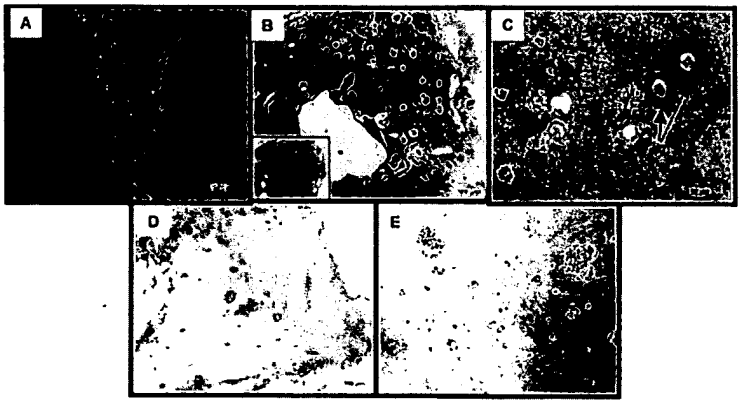

Figure 6. Blood vessel growth in the subcutis/HBMSC encapsulated alginate-chitosan implants (A). vWF immunohistochemistry of HBMSC with and without labelled particles encapsulated in alginate/chitosan capsule implanted subcutaneously in MF-1 nu/nu mice and exposed to a magnetic field for 1hr a day (Monday, Wednesday, Friday) for 21 days. 6μm tissue sections stained for vWF; HBMSC only capsules (B&C), HBMSC labelled TREK-K^{+} particle capsules (D), HBMSC labelled RGD particle capsules (E).